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ONCOSTATIN M

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Oncostatin M (OSM) was initially identified as a polypeptide cytokine which inhibited the in vitro *growth of cells from melanoma and other solid tumors. OSM shows significant similarities in primary amino acid sequence and predicted secondary structure to leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), granulocyte colony-stimulating factor (G-CSF), interleukin 6 (IL-6), and interleukin 11 (IL-11). Analysis of the genes encoding these proteins reveals a shared exon organization suggesting evolutionary descent from a common ancestral gene. Recent data indicates that OSM also shares a number of* in vitro *activities with other members of this cytokine family. The overlapping biological effects appear to be explained by the sharing of receptor subunits.*

Keywords: Leukemia inhibitory factor, granulocyte colony-stimulating factor, ciliary neurotrophic factor, interleukin 6, interleukin 11, growth hormone, receptors, hematopoietins

INTRODUCTION

Oncostatin M (OSM) was initially described in 1986 as a growth regulatory molecule which could inhibit the growth of certain tumor cell lines and stimulate the growth of several normal fibroblast lines [1]. Recently, OSM has been recognized as a member of a growing family of pleiotropic cytokines which includes leukemia inhibitory factor (LIF), granulocyte colony-stimulating factor (G-CSF), ciliary neurotrophic factor (CNTF) and interleukin 6 (IL-6) [2,3]. A wide variety of biological activities have been attributed to members of this cytokine family which suggests that they play important roles in hematopoiesis, neuropoiesis, bone remodeling, cachexia, inflammation, muscle proliferation and embryonic development [for reviews see 4–6]. Although unique biological functions have been associated with individual members of the family, it is becoming clear that these cytokines share many common functions. This review will

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focus on OSM and its structural and functional relationships with other members of the cytokine family.

PROTEIN STRUCTURE OF OSM

OSM was originally isolated from media conditioned by U937 human histiocytic leukemia cells induced to differentiate into macrophage-like cells by treatment with phorbol 12-myristate 13-acetate (PMA) [1]. A partial amino acid sequence was obtained which allowed the isolation of cDNA and genomic clones for human OSM using degenerate oligonucleotides [7]. The human OSM gene encodes a 252 amino acid precursor polypeptide. N-terminal sequence analysis of the isolated protein indicated that the first 25 amino acids function as a signal peptide which is subsequently removed during the post-translational secretory process, yielding a 227 amino acid pro-OSM. In addition, the 31 residues encoded at the carboxy-terminus of the pro-OSM are removed by cleavage at a trypsin-like site (RSRR) resulting in a 196 amino acid mature form [8]. The 196 amino acid OSM is the predominant form isolated from PMA-treated U937 cells and from expression of the human cDNA in CHO cells [8]. The OSM sequence contains two potential *N*-linked glycosylation sites and sites for *O*-linked glycosylation. The mature form of OSM migrates in SDS-PAGE with a $M_r \sim 28{,}000$ daltons. The 227 amino acid pro-OSM has also been isolated from COS cells and is as active as a 196 residue mature OSM in competition binding assays, but was 5- to 60-fold less active in growth inhibition assays [9]. Although the 227 amino acid pro-OSM has not yet been found to occur naturally, it could have a distinct biological role. Thus, the regulation of post-translational processing of OSM may be important for its *in vivo* function.

The mature OSM is predicted to adopt a four alpha-helical bundle structure similar to that determined for growth hormone [2,3]. The helical domains of OSM are amphipathic with a repeated pattern of apolar residues occurring in the i and $i+3$ positions of 7-residue heptads. This pattern of apolar residues within helical regions is implicated in the interactions between alpha helices [10]. Mutational analysis demonstrated that discontinuous regions, including the exceptionally amphipathic C-terminal helix, are involved in receptor binding [11]. OSM contains five cysteine residues with four of them forming two intramolecular disulfide bonds as shown in Fig. 1 [11]. The disulfide bond between the second and fifth cysteine residues links helix 4 with the turn region between helix 1 and 2 and is necessary for the activity of OSM [11]. The disulfide linkage between first and fourth cysteine residues is not required for biological activity [11]. The active form of the molecule is thought to be a monomer with no observed intermolecular disulfide bonding through the free cysteine residue.

GENE STRUCTURE OF OSM

Analysis of OSM cDNA clones derived from human U937 histiocytic leukemia cells treated with PMA revealed an open reading frame of 784 nucleotides which encodes the 252 amino acid OSM precursor [7]. Northern analysis of poly A mRNA isolated from treated U937 cells demonstrated that the OSM mRNA is approximately 2,000 bp [7]. 25 nucleotides of 5′ non-coding and 1,055 nucleotides of 3′ non-coding sequences

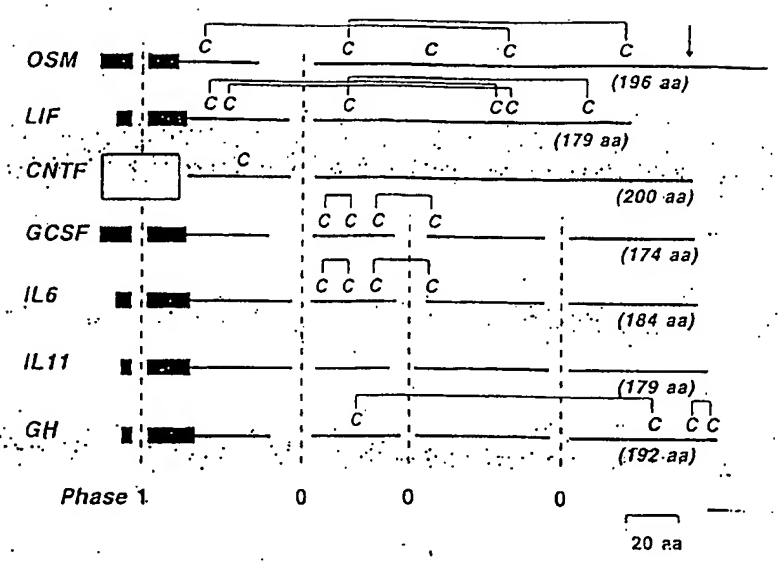

FIGURE 1. Schematic representation of the exon boundaries within the protein precursors for human OSM [7], LIF [41], CNTF [42], G-CSF [43], IL-6 [44], IL-11 [45] and GH. The solid lines represent the mature polypeptides and the shaded boxes represent the signal sequences. An arrow marks a proteolytic cleavage site within the C-terminal region of OSM [9]. The sizes of the mature polypeptides are indicated. The positions of conserved cysteine residues are noted (C) and the known disulfide linkages are shown. The exon boundaries are denoted with dashed vertical lines and the phasing of the junction is given.

have been identified in cDNA clones. However, no poly A tract was detected in the cDNA clones, nor was a polyadenylation consensus signal (AATAAA) identified in either the cDNA clones or the subsequent 500 nucleotides of the 3′ untranslated region present in the genomic clone (unpublished results). These results suggest that a sequence different from the consensus AATAAA polyadenylation signal establishes the 3′ end of the mRNA. The 3′ end of the OSM cDNA contains an A + T rich region with several ATTTA pentamer motifs and a TTATTTAT octamer motif which is involved in the regulation of the stability of many cytokine and lymphokine transcripts [12].

The expression of OSM mRNA is generally restricted to hematopoietic cells including phytohemagglutinin-activated peripheral blood T lymphocytes (13) and lipopolysaccharide-induced human monocytes (unpublished observation, K. Grabstein and T. Rose). The expression of OSM mRNA is induced in U937 histiocytic leukemia cells upon treatment with the phorbol-ester (PMA) [7]. The only known non-hematopoietic source of OSM RNA is cells derived from AIDS-related Kaposi's cells [14].

Three exons containing the entire coding and non-coding sequences of OSM were identified from a genomic clone derived from human brain tissue [7]. The intron–exon junction sequences conform to the GT...AT rule for nucleotides flanking eukaryotic exon boundaries. The first exon contains 5′ non-coding DNA and the DNA encoding the initiating methionine and 10 amino acids of the signal sequence. The second exon

encodes the remainder of the signal sequence and the first 34 residues of the mature molecule. The last exon encodes the remaining 193 amino acids and contains all of the 3′ non-coding sequences present in the cDNA clone. The entire human OSM gene spans approximately 5 kb.

IN VITRO EFFECTS OF OSM

The *in vitro* biological activities presently attributed to OSM are summarized in Table 1. As with most cytokines, these activities are diverse and, in some cases, appear contradictory.

TABLE 1. Summary of *in vitro* properties of OSM.

Solid tumor cells
Inhibits the growth of a solid tumor cells from a wide variety of sources [1, 15].
Hematopoietic cells
Induces the differentiation of human and murine leukemia cells [2, 16].
Embryonal cells
Blocks the differentiation of totipotent mouse embryonic stem cells [24].
AIDS-related Kaposi's sarcoma cells
Is produced by AIDS–KS cells and acts in autocrine and paracrine fashion as a mitogen for these cells [14, 26].
Stimulates the production of IL-6 in these cells [14].
Hepatic cells
Induces acute phase protein synthesis in HepG2 cells [31].
Induces an increase in the number of LDL receptors on HepG2 cells [36, 37].
Fibroblasts
Stimulates the production of urokinase-type plasminogen activator in synovial fibroblast-like cells from rheumatoid lesions [39].
Stimulates the growth of several fibroblast cell lines [1, 15].
Endothelial cells
Induces the production of IL-6 in human umbilical vein endothelial cells [27].
Inhibits the growth of bovine aortic endothelial cells and stimulates the cells to produce plasminogen activator [40].

Cell Lines from Solid Tumors

Treatment of a number of human tumor cell lines derived from a wide variety of tissue types with recombinant OSM leads to the inhibition of proliferation and changes in cellular morphology [1, 15]. The majority of tumor cell lines tested show significant reduction in growth in response to OSM treatment with a half-maximal response obtained at concentrations of 1–50 pM. Inhibition of proliferation was detected in a high proportion of lung carcinoma (5/6) and breast carcinoma (2/3) cell cultures. Tumor lines from six colon and two prostate carcinomas were the exception where no response was detected even though receptors were present in most cases [15].

Leukemia Cell Lines

OSM induces the differentiation of murine M1 myeloid leukemia cells, an activity shared with a number of cytokines including LIF, IL-6 and G-CSF [2, 16]. Treatment with OSM induces the M1 cells to take on characteristic macrophage-like phenotypes, such as a reduction in the nucleus/cytoplasm ratio, the presence of vacuoles in the

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cytoplasm and phagocytosis of *E. coli*. OSM also causes an increase in the number of dispersed colonies in soft agar, another indication of the presence of the more mobile macrophage-like cells.

OSM induces the differentiation of human U937 histiocytic leukemia cells in the presence of GM-CSF [16], as shown for LIF and IL-6 [17, 18]. Incubation of U937 cells with OSM alone does not cause significant changes in colony morphology in soft agar. However, in combination with GM-CSF, OSM causes a significant increase in the proportion of dispersed colonies, an indication of differentiation into macrophage-like cells.

Embryonic Stem Cells

Maintenance of the undifferentiated pluripotent phenotype of embryonic stem (ES) cells *in vitro* is dependent upon the presence of either a feeder layer of fibroblasts or conditioned media obtained from cultured tumor cells [19,20]. The feeder layer or conditioned medium can be replaced by the addition of purified LIF [21,22]. ES cells grown in the presence of LIF will retain their characteristic morphology and the ability to express stem cell-specific surface antigens, such as those recognized by the ECMA-7 monoclonal antibody [23]. The ES cells maintained in LIF can be implanted into mouse blastocysts to produce chimeric mice. In culture, removal of LIF causes the ES cells differentiate into a variety of cell types with distinct morphological characteristics. The differentiated cells no longer express stem cell-specific antigens [21,23]. Recently, OSM has also been shown to inhibit the differentiation of ES cells cultured *in vitro* [24]. The results with OSM were equivalent to those obtained with LIF. The totipotent nature of the cells grown in the presence of OSM has not yet been demonstrated *in vivo*, however, these results implicate OSM as a developmental regulatory factor for embryonic stem cells.

Kaposi's Sarcoma Cells

Aids-related Kaposi's sarcoma (AIDS–KS) is a malignant, neoplastic proliferation of mesenchymal cells and is the most common malignancy found in patients infected with human immunodeficiency virus (HIV) [25]. AIDS–KS derived cells have been shown to produce OSM which then acts in autocrine and paracrine fashions as a potent mitogen for these cells [14]. OSM also appears to be required for the long-term maintenance of these cells *in vitro* [26]. OSM, interleukin 1 (IL-1) and tumor necrosis factor alpha (TNFα) induce the production of IL-6 in AIDS–KS cells [14], similar to their effects on endothelial cells [27–30]. Since IL-6 is a mitogen for AIDS–KS cells, it was necessary to determine whether the effect of OSM on these cells is caused by induction of IL-6 as is the case for IL-1 and TNFα. The activity of OSM appears to be independent of IL-6 induction since antisense IL-6 oligonucleotides, which blocked the effects of IL-1 and TNFα, did not block the proliferative effects of OSM [14]. The findings that OSM is necessary for the maintenance of the cellular phenotype and for the continued proliferation of AIDS–KS cells suggest that OSM may contribute to the pathogenesis and progression of Kaposi's sarcoma.

Hepatic Cells

During acute inflammation, there is a significant change in the plasma levels of a number of liver-derived proteins referred to as acute phase proteins (APP). These proteins are induced by the actions of several cytokines on hepatocytes. OSM induces the same set of APP (type II) in human hepatoma HepG2 cells [31] as IL-6 [32,33], IL-11 [34] and LIF [35], however, the level of induction with OSM was greater than with the other cytokines [31]. These results suggest that OSM may play a role in regulating inflammatory responses.

The low density lipoprotein (LDL) receptor plays an important role in regulating the level of serum cholesterol. OSM acts on HepG2 cells to induce an increase in the number of LDL receptors [36, 37]. This increase in receptor number causes an increase in the uptake of LDL-derived cholesterol which is thought to elevate intracellular free cholesterol levels and decrease transcription for key enzymes involved in the cholesterol pathway. The potential clinical significance of this result is demonstrated by a recent study where similar effects on hepatic LDL receptor levels were observed *in vivo* when humans were treated with human growth hormone [38], a cytokine related to OSM (see below). The growth hormone treatment resulted in a 25% decrease in serum cholesterol levels, comparable to the effects of an established hypolipidemic drug (pravastatin or simvastatin). These results suggest that OSM may also be involved in regulating serum cholesterol levels.

Fibroblasts

In contrast to tumor cell lines, fibroblasts from a variety of sources are induced to proliferate when treated with OSM [1, 15]. Activated synovial fibroblast-like cells, which are invasive cells present in inflamed synovium of rheumatoid lesions, are stimulated by OSM to produce urokinase-type plasminogen activator (u-PA) [39]. U-PA, which plays a role in the process of connective tissue turnover and inflammation by activation of the serine protease plasmin, is found in elevated levels in rheumatoid tissues and synovial fluids [39]. Vascular endothelial cells also show an increase in plasminogen activator levels in response to OSM treatment [40]. These results suggest possible roles for OSM in regulation of vascular fibrinolysis and the pathogenesis of rheumatoid arthritis.

RELATIONSHIP TO OTHER CYTOKINES

Gene Structure Comparison

OSM shares amino acid sequence similarities, a common secondary structure and a similar gene structure with the cytokines leukemia inhibitory factor (LIF), granulocyte colony-stimulating factor (G-CSF), interleukin 6 (IL-6) and ciliary neurotrophic factor (CNTF) suggesting that these cytokines evolved from a common ancestral gene [2, 3]. By similar criteria, these cytokine genes are also more distantly related to the growth hormone (GH) gene, and a number of other genes including prolactin, erythropoietin and interleukin 7 [2, 3]. As shown in Figs 1 and 2. the gene for interleukin 11 (IL-11) can also be included as a member of this family of growth regulatory proteins.

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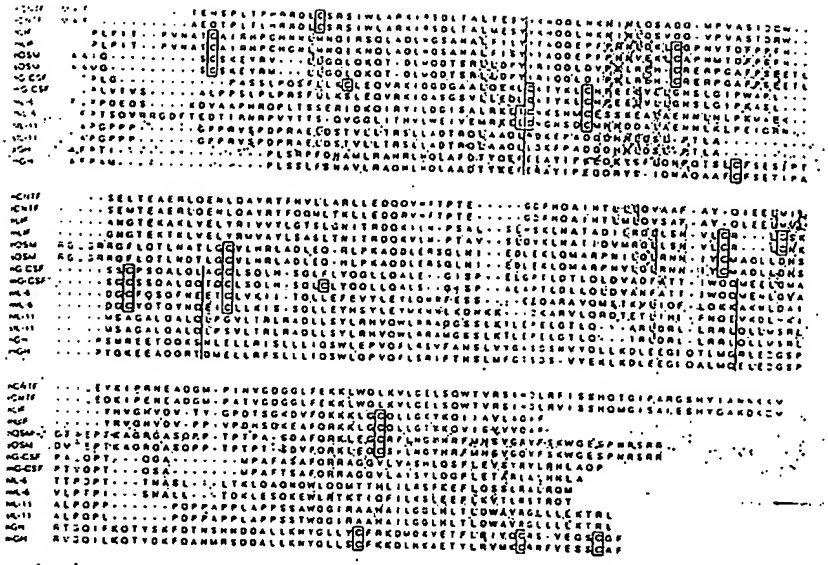

FIGURE 2. Amino acid sequence alignment of the mature polypeptides of the human and simian homologues of OSM [7 and unpublished results], the human and rat homologues of IL-11 [45, 46] and the human and murine homologues of LIF [41, 47], CNTF [42, 48], G-CSF [43, 49], and IL-6 [44, 50]. Exon boundaries are denoted with a vertical line. Residues occurring in two or more of the six different gene families (OSM, LIF, CNTF, G-CSF, IL-6 and IL-11) are shaded and the cysteines are boxed. Unknown residues within the simian OSM sequence are denoted (.).

Pictographs of the gene structures for human OSM, LIF, CNTF, G-CSF, IL-6, IL-11 and GH are shown in Fig. 1. OSM and LIF have identical exon organizations with three exons separated by two introns. The phasing of the analogous junctions are identical in both genes. The gene for CNTF does not encode a signal peptide and lacks the first intron found within the region encoding the signal peptide in the OSM and LIF genes. However, the CNTF gene has an intron in a position analogous to the second intron of both the OSM and LIF genes and the splice junction maintains the same phasing.

In addition to the junctions found in OSM, LIF and CNTF, the genes for G-CSF, IL-6, IL-11 and GH contain two additional intron–exon junctions within the region of the final exon of OSM, LIF and CNTF. The phasing of both of these junctions is identical in each gene. As indicated in Fig. 1, the sizes of the corresponding exons of the seven different genes are quite similar, as are the sizes of the final mature peptides.

Amino Acid Sequence Alignment

An alignment of the amino acid sequences encoded by the different family members is presented in Fig. 2. The analogous exon boundaries within the mature proteins were aligned and the amino acid sequence alignment was optimized. Significant amino acid similarity is detected between every sequence throughout their entire coding regions and obvious conserved sequence patterns are observed. The greatest amino acid

similarities are detected between OSM and LIF, and between OSM and G-CSF with approximately 22–29% identical residues [2]. When the conservative substitution of like amino acids is allowed, the sequence similarity rises above 30%. The most important conserved motif is found within the C-terminal regions of the cytokines and contains the sequence pattern A/V–F–Q–K/R–K/R–X–X–G–C/V. Human and simian OSM and the human and murine homologues of LIF and G-CSF contain this motif. In the other sequences the aromatic nature of the second residue, Phe (F), is conserved, with a Phe (F) residue present in CNTF, a Trp (W) residue present in IL-6 and IL-11, and a Tyr (Y) residue present in HG. Another notable sequence motif (F/Y–L/M) is also present in the C-terminal region of OSM, G-CSF, IL-6 and GH. Although the overall sequence similarities between members of this family is low (10–29%), similar conserved motifs are observed throughout the entire coding regions of the sequences. Secondary structure analysis of the primary amino acid sequences for each cytokine predicts extensive alpha-helical configurations and each of the cytokines is predicted to form a four alpha-helical bundle patterned after the structure determined for growth hormone by X-ray crystallography [51].

Chromosomal Localization

The gene for OSM has been localized to chromosome 22 in the human genome. The gene for LIF has also been localized to chromosome 22 at 22q12 (52, 53). The finding that these two related genes are present on the same chromosome further suggests a common evolutionary origin for these genes.

Receptors

Receptors for OSM are found on cells from a wide variety of sources [15, 54]. In general, cells from tumor and endothelial origins have high levels of OSM receptors and cells from hematopoietic origins have low numbers. Scatchard analysis of the binding of OSM to A375 melanoma cells and A549 lung carcinoma cells revealed a curvilinear pattern indicating the presence of more than one receptor: a low affinity form, $K_d \sim 500$ pM and a high affinity form $K_d \sim 5$ pM [54]. Cross-linking experiments with ^{125}I-OSM showed that the majority of binding was to a 160kDa protein [54]. Recently, the cross-linked protein was identified as gp130, the IL-6 signaling subunit [55]. Additional studies have shown that gp130 is the low affinity form of the OSM receptor [55,56] and anti-gp130 antibodies block the growth inhibitory effects of OSM [55]. Gp130 is also part of both the LIF [56] and CNTF [57] receptors. In addition, gp130 also appears to be part of the IL-11 receptor since IL-11 cross-links to a protein approximately the size of gp130 [58].

A current model for the receptors of the family members is shown in Fig. 3. In this model, all the high affinity receptor complexes consist of combinations of receptor subunits which are related to each other and belong to an extended family of "hematopoietin" receptor molecules [59, 60]. Ligand specificity is conferred by a low affinity interaction with an "alpha" receptor subunit. The low affinity binding is not thought to be sufficient to trigger a biological response. Interaction with additional subunits creates the high affinity form of the receptor. In our model, each receptor complex includes two subunits whose cytoplasmic domains interact to allow signal transduction. Where the alpha subunits have little or no cytoplasmic domain, the complexes include at least two other subunits with cytoplasmic signaling domains.

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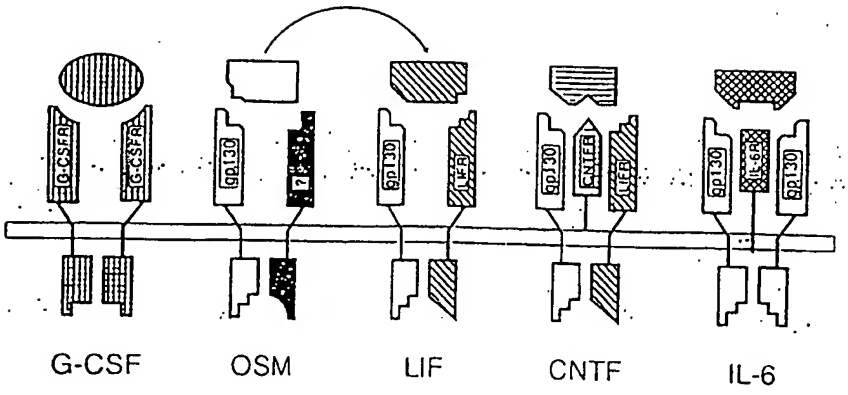

FIGURE 3. A model for the cytokines binding to their high affinity receptor complexes. For each cytokine, the shading matches the "alpha" subunit which it has been shown to bind directly with low affinity. A second subunit for the OSM receptor complex is shown but has not yet been identified. The arrow indicates that, in addition to binding the OSM receptor, OSM is able to bind to the LIF receptor.

In the simplest case, the high affinity form of the G-CSF receptor is a homodimer of the G-CSF receptor subunit (GCSFR) [61]. In this case, GCSFR is responsible for both the specificity and signaling. The high affinity LIF receptor is structurally similar to the G-CSF receptor except it is a heterodimer of the 210 kDa LIF alpha subunit (LIFR), which binds LIF directly with low affinity, and gp130 [56]. Both of these molecules have significant homology with GCSFR [62]. The high affinity form of the CNTF receptor, like the LIF receptor, includes single gp130 and LIFR subunits plus a separate alpha subunit (CNTFR) which binds CNTF directly with low affinity [57, 63]. The high affinity IL-6 receptor consists of the IL6R alpha subunit, which binds IL-6 directly with low affinity, and gp130 [64]. IL-6R has significant homology with CNTFR [63] and like CNTFR, lacks a cytoplasmic signaling domain. It has been proposed that the IL-6 receptor complex contains two gp130 subunits to make it consistent with the other family members [57], however, this has not been demonstrated experimentally.

OSM binds directly to its alpha subunit, gp130, with low affinity [55, 56]. The remaining subunit(s) which interacts with gp130 to create the high affinity form of the OSM receptor has not yet been identified. It is unlikely that the OSM receptor is a simple homodimer of gp130 since the high level expression of gp130 does not result in an increase in the number of the high affinity OSM receptors [56]. It seems more likely that the OSM receptor is structurally similar to the LIF receptor with gp130 as the alpha subunit and a second subunit which is probably related to gp130, LIFR and GCSFR and has a cytoplasmic signaling domain.

Human OSM competes with human LIF for binding to high affinity murine LIF receptors and chimeric LIF receptors on murine B9 cells expressing human LIFR [65]. In both cases, human LIF binds with an affinity about five-fold higher than human OSM. Human OSM does not bind directly to the human LIFR subunit which binds human LIF with low affinity [65]. Receptors which bind OSM but not LIF are present in significant numbers on several human cell lines, but not on any of the murine cell lines tested to date [16, 24, 65]. The biological effects of human OSM on murine cells described so far all appear to be mediated through the LIF receptor. The failure to

detect OSM specific receptors on murine cells may reflect the absence of a murine OSM receptor or that the binding of human OSM to the murine OSM receptor is too weak to detect. The ability of OSM to bind the high affinity LIF receptor suggests that OSM may have all the *in vitro* effects of LIF but, since some cells have only OSM specific receptors, OSM probably has additional activities.

Signal Transduction

The effect of OSM on the early-immediate genes *EGR-1*, c-*jun* and c-*myc* has been investigated in several cell types: human fibroblasts which are induced to proliferate, murine M1 leukemic cells which are induced to differentiate and human A375 melanoma cells which are growth inhibited by treatment with the cytokine [66]. Treatment of fibroblasts and M1 cells with OSM results in transient increases in the mRNA levels of the early-immediate genes during the first 2–3 h. Since M1 cells do not appear to have OSM specific receptors, the effects of OSM are probably through interactions with the LIF receptor [65]. In fact, similar results on M1 cells treated with LIF have been observed [67]. It is not known which receptors are responsible for the effects on fibroblasts. A375 melanoma cells, which have OSM specific receptors but lack LIF receptors [65], showed no change in EGR-1, c-jun or c-myc mRNA levels when treated with OSM. Growth inhibition of A375 cells also appears to be independent of protein kinase C [66], however, protein kinase C stimulation appears to be important for some of the effects of OSM, such as LDL-receptor induction in HepG2 cells [37].

OSM, LIF, CNTF, IL-6 and IL-11 induce the phosphorylation of a ~ 160 kDa protein [54, 57, 58, 64]. With OSM and IL-6, this protein has been identified as gp130 [55, 63]. The phosphorylation of a tyrosine on the cytoplasmic portion of gp130 is required for IL-6 signaling activity [68]. The activation of tyrosine kinases by OSM was studied in human endothelial cells which are stimulated to produce IL-6 by treatment with OSM [69]. These cells have high levels of OSM specific receptors but lack LIF receptors [65]. Pretreatment with tyrosine kinase inhibitors genistein and herbimycin A blocks the induction of all OSM specific tyrosine phosphorylation but not of constitutively phosphorylated proteins. The inhibitors also block the induction of IL-6 synthesis suggesting that this phosphorylation is essential for the effect of OSM on these cells. Because herbimycin A is known to inhibit src family kinases, the effects of OSM on these kinases were determined. OSM increases the activity of the $p62^{yes}$ and, to a lesser extent, $p59^{fyn}$. The diverse nature of the effects of OSM and the other members of the family may reflect differences in substrates available for phosphorylation or different kinases associated with the receptors in different cell types.

SUMMARY

A number of *in vitro* activities have been found for OSM which suggest potential *in vivo* roles in hematopoiesis, inflammation, cholesterol regulation and embryonic development. The ability of OSM to act through the LIF receptor suggests that, like LIF, OSM may also play a role in bone growth, lipid metabolism and neuronal development [4]. The extent of the functional overlap between OSM and LIF is not known, although OSM clearly has activities which are different from LIF such as the ability to inhibit the growth of A375 and H2981 tumor cells [65].

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